

The Chemiluminescent Response from Human Monocyte-Derived Macrophages Exposed to Various Mineral Fibers of Different Sizes

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Abstract: The aims of the present work were to quantify ability to induce lucigenin-dependent chemiluminescence (CL) from 6–9 day-old human monocyte-derived macrophages exposed to various mineral fibers, and to examine the relationship between ability to induce CL and fiber size. All fiber samples induced the CL response from the cells. The relationship between the number of fibers administered and the CL response was examined on all fiber samples by linear regression. The slope of the regression line supplies an approximation of the ability to induce CL. A strong increased correlation between geometric-mean length of fibers and ability to induce CL was observed for the seven fiber samples more than 6 μm in length ($r=0.9895$). Geometric-mean width and the ability to induce CL showed no correlation. However, among the two fiber samples having a similar length distribution (RF2, RF3), the wider width sample (RF3, 2.4 μm) demonstrated lower ability to induce the CL than the narrower width sample (RF2, 1.1 μm). The present method enabled comparison of ability to induce lucigenin-dependent CL from human monocyte-derived macrophages for various mineral fibers with different sizes. Our findings suggested the possibility that ability to induce O_2^- production increased with fiber length, when fibers are longer than approximately 6 μm .

Key words: Man-made mineral fibres, Reactive oxygen, Superoxide, Macrophage, Monocyte, Lucigenin, Fiber size, Fiber length

Introduction

Exposure to asbestos is associated with the development of mesotheliomas, lung cancers and fibrotic lung diseases¹. Therefore, man-made mineral fibers (MMMf) have recently been used for asbestos substitution. Epidemiological studies of the carcinogenicity of MMMf have found inconclusive². In the animal intraperitoneal studies, however, fiber length has been found to be one of the major descriptors of tumorigenicity^{3–5}.

It has been hypothesized that the cytotoxic, genotoxic and proliferative effects of asbestos are in part mediated by the

production of reactive oxygen species released by alveolar macrophages in response to engulfment of long fibers⁶. Long chrysotile asbestos fibers have been found to be more effective than short fibers in eliciting release of O_2^- from rat alveolar macrophages⁷. Moreover, for amosite asbestos with opsonization, a dramatic enhancement of release of O_2^- has been found with long fibers, but not short ones⁸. Among various mineral particles, fibrous dusts have been found to cause a significant increase in release of O_2^- from macrophages^{9–11}, and nonfibrous particles were less active than fibers¹². Nevertheless, the relationship between fiber length and ability to induce O_2^- production from macrophages is still unclear for various mineral fibers. The geometry of MMMf resembles that of asbestos, and oxidants play an

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important role in asbestos-related pulmonary disease. The purposes of the present study were to quantify ability to induce lucigenin-dependent chemiluminescence (CL) from 6–9 day-old human monocyte-derived macrophages exposed to various mineral fibers, and to examine the relationship between ability to induce CL and fiber size.

Materials and Methods

Mineral fibers

We used the Japan Fibrous Material (JFM) standard reference samples provided by the Japan Fibrous Material Research Association (JFMRA), glass wool (GW1, Geometric-mean length: 20.0 μm , Geometric-mean width: 0.88 μm , number of fibers per unit weight: $0.7 \times 10^3/\mu\text{g}$), rock wool (RW1, 16.5, 1.8, 1.7), micro glass fiber (MG1, 3.0, 0.24, 65), refractory ceramic fiber (RF1, 12.0, 0.77, 8.8), (RF2, 11.0, 1.1, 8.7) refractory mullite fiber (RF3, 11.0, 2.4, 3.5), potassium titanium whisker (PT1, 6.0, 0.35, 590), silicon carbide whisker (SC1, 6.4, 0.30, 410), titanium oxide whisker (TO1, 2.1, 1.00, 640), and wollastonite (WO1, 10.5, 1.00, 24). Characterization of these fibers has documented elsewhere^{13,14}. Each sample was dried and heat-sterilized at 80°C for 48 hours, and suspended in Hanks' balanced salt solution (HBSS) at 5 concentrations of 10 mg/ml or less, except for SC1, which was adjusted to 1/10 the concentration of other samples. These suspensions were stored at 4°C.

Cell isolation

Heparinized blood was obtained from healthy donors by venipuncture and diluted 1:1 in HBSS. Monocyte-lymphocyte fractions were isolated by Ficoll density centrifugation and plated in 9-cm-diameter plastic tissue culture dishes for monocyte adherence¹⁵. The adhering cells were cultured for 9 days in RPMI1640 with 10% fetal bovine serum (FBS), 100 U penicillin/ml, and 100 μg streptomycin/ml. This culture medium was changed every 2 days. Adherent cells were separated after 6 days, and suspended in serum free RPMI1640.

Chemiluminescence measurements

The measurement of lucigenin-dependent CL from 6 day-old human monocyte-derived macrophages exposed to various mineral fibers has previously been described: the lucigenin responses increased with increasing age of cultures during 6 days, and a correlation was between lucigenin-dependent CL and O_2^- production measured with the cytochrome C reduction assay at 6 days of culture⁹.

The isolated cells (1×10^5 cells) were transferred into a

luminometer tube containing mineral sample suspension (65 μl), 10% FBS, 0.1 mM lucigenin, and in some experiments 1000 unit/ml superoxide dismutase (SOD). The final volume of each tube was 1 ml. The light emission of each sample was detected at 15-min intervals with a luminescence reader (ALOKA BLR-201). All samples including negative control (no fiber) were measured with the same cell suspension in 10-second intervals. All reactions were performed at 37°C in RPMI 1640. This measurement was performed 4 times.

Statistical analysis

The relationship between the number of fibers administered and CL response (average of 4 times) was examined by linear regression, with the following equation:

$$Y = \beta_0 + \beta_1 X$$

Y: CL, X: estimated number of fibers, β_0 , β_1 : constant

The number of fibers administered was calculated using the number of fibers per unit weight¹⁴ and the sample weight administered. The slope (β_1) was taken as a measure of ability to induce CL. The relationship between fiber size and ability to induce CL was examined by linear regression with samples with values of $r^2 > 0.8$.

Results

CL response was detected from 6–9 day-old cells in the lucigenin following the administration of each sample under 650 $\mu\text{g}/\text{ml}$. Each CL response was almost completely inhibited by SOD, which is a superoxide scavenger (data not shown).

Fig. 1 shows the time-dependence of CL response of isolated cells to JFM standard reference samples with similar estimated numbers of fibers. The results of GW1, RW1, RF1, and RF2 were similar. RF3 and WO1 appear to be weaker CL response than these four samples. The CL response of SC1 could not be observed. Various CL responses were demonstrated even with similar estimated numbers of fibers.

The relationship between estimated numbers of fibers and CL response at 45 minutes is shown in Fig. 2. The straight lines are regression lines. Constants and r^2 values are shown in Table 1. WO1 demonstrated the lowest r^2 values, 0.12, while other samples had r^2 values exceeding 0.8.

Fig. 3 shows the relationship between geometric-mean length and β_1 . A close correlation existed between length and β_1 , on the whole ($r=0.9540$), although four samples under approximately 6 μm in length (SC1, PT1, MG1 and TO1) demonstrated low β_1 values. For seven samples longer than 6 μm (GW1, RW1, RF1, RF2, RF3, SC1 and PT1), a strong

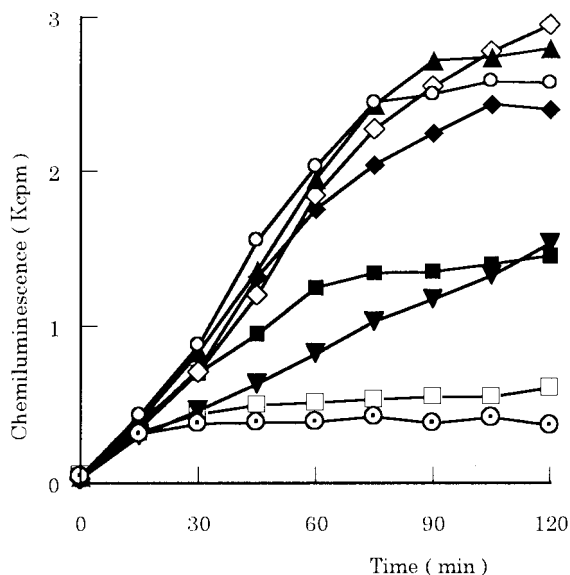


Fig. 1. The time-dependence of CL response of human monocyte-derived macrophages to JFM standard reference samples with similar estimated numbers of fibers (n=4).

SC1, estimated dose number of fibers $20.7 (\times 10^5)$ numbers of fibers/ml; RF3, 7.0; WO1, 12.15; \blacklozenge RF2, 4.35; \blacklozenge RF1, 4.4; RW1, 3.4; GW1, 4.55; \bullet Control, no fiber.

correlation existed between length and β_1 ($r=0.9895$).

Fig. 4 shows the relationship between geometric-mean width and β_1 . No correlation was observed between these ($r=0.4492$). On comparison of fibers having a similar length distribution, however, the $2.4 \mu\text{m}$ width fiber (RF3) demonstrated lower β_1 than $1.1 \mu\text{m}$ width fiber (RF2).

Discussion

We quantified lucigenin-dependent CL from cells exposed to different sizes of various mineral fibers, and examined the relationship between CL and fiber size. For quantification of ability to induce CL per fiber, the relationship between CL response and sample dose was examined by linear regression. The slope of the regression line supplies an approximation of the ability to induce CL per fiber.

This method enabled the comparison of ability to induce CL among samples of different optimum density. For example, CL response of SC1 was not observed with optimum densities of RW1 or GW1, as shown in Fig. 1. However, the CL response of RW1 or GW1 was saturated with optimum density of SC1. Therefore, comparison of ability to induce CL by this method may be more accurate than comparison by experiments with equal numbers of fibers. In this method,

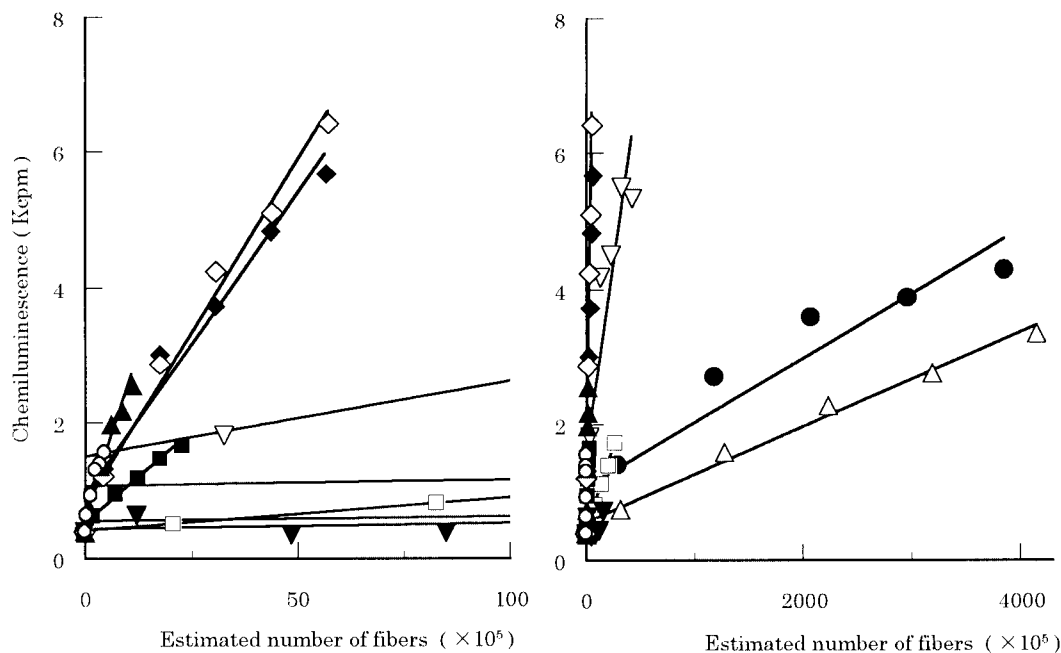
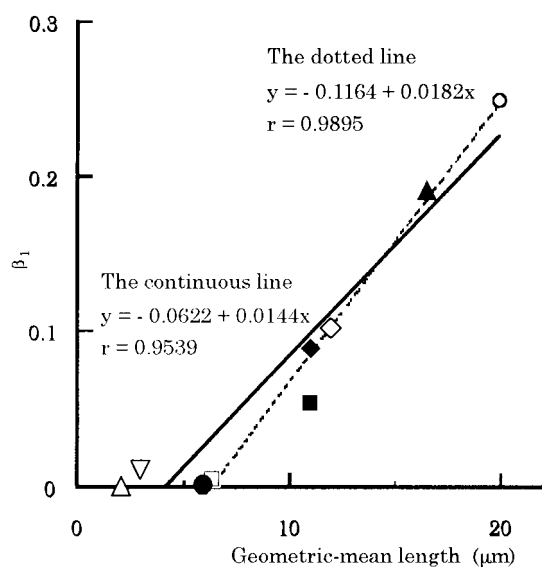


Fig. 2. The CL response of human monocyte-derived macrophages to JFM standard reference samples at 45 minutes (n=4).

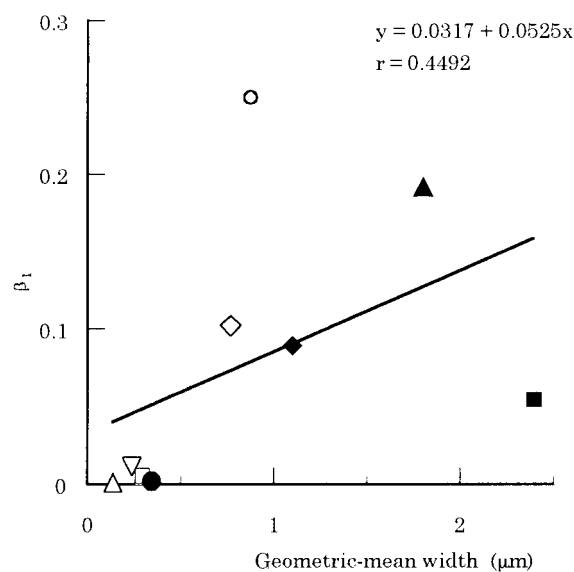
GW1; RW1; \blacklozenge RF1; \blacklozenge RF2; RF3; WO1; SC1; TO1; MG1; PT1. The left figure shows under 10 million of the right figure horizontal axis.

Table 1. Constants and r^2 of the regression lines for CL and estimated number of fibers

	PT1	TO1	MG1	RF1	RF2	RF3	WO1	GW1	RW1	SC1
β_0	1.0522	0.5385	1.4912	0.7477	0.8918	0.4986	0.4203	0.5238	0.5855	0.3975
β_1	0.0010	0.0007	0.0112	0.1021	0.0894	0.0539	0.0009	0.2492	0.1920	0.0049
r^2	0.8962	0.9876	0.8177	0.9848	0.9669	0.9788	0.1237	0.9424	0.9607	0.9996

**Fig. 3.** The relationship between geometric-mean length and β_1 .

Symbols as in Fig. 2. The continuous line is a regression line in nine samples, excluding WO1. The dotted line is a regression line in seven samples over 6 μm in length.

**Fig. 4.** The relationship between geometric-mean width and β_1 .

Symbols as in Fig. 2. The geometric-mean length of RF2 (\blacklozenge) and RF3 (\square) is 11.0 μm . The continuous line is a regression line in nine samples, excluding WO1.

it is important that the relationship between CL response and estimated number of fibers be fit by straight line.

However, the sizes of samples tested differed. For example, long glass fibers (GW1) had a larger ability to induce CL than did short ones (MG1). The difference in ability to induce CL between GW1 and MG1 was considered to depend on size. Therefore, the relationship between size and ability to induce CL was examined. However, WO1 with low r^2 was excluded.

The ability to induce CL increased with fiber length, for fibers longer than approximately 6 μm (Fig. 3). There was no correlation between width and slope of the regression line, however; RF3, the thickest sample, may induce CL only weakly (Fig. 4). Our findings suggested the possibility that ability to induce O_2^- production increased with fiber length, when fibers are longer than approximately 6 μm .

Previous study with various mineral particles has suggested that the fibrous geometry of particulates is of critical importance in the generation of O_2^- from macrophages¹².

We found further detailed relationship between fiber length and elicitation of release of O_2^- from macrophages.

Long asbestos fibers have been found to be more effective than short fibers in eliciting release of O_2^- from macrophages^{7,8}. For example, for amosite asbestos with opsonization, dramatic enhancement of release of O_2^- has been found with long fibers, but not short ones⁸. The distribution of length of the long fibers is similar to that of RF1 (mean length 12.0 μm), and the distribution of length of the short fibers is similar to that of MG1 (mean length 3.0 μm). Therefore, Our data of the relationship between fiber length and ability to induce CL was consistent with the asbestos data.

Murine peritoneal macrophages exposed to equal numbers of short and long crocidolite asbestos fibers exhibited comparable H_2O_2 release¹⁶. The mean length of the long crocidolite was 5.4 μm , and the mean length of short one was 1.2 μm . Since O_2^- is also related to H_2O_2 generation, our result that ability to induce CL was similar with under

approximately 6 μm in length was consistent with the H_2O_2 data.

Not only fiber size but also durability is important in determining the biological effects of various mineral fibers *in vivo*¹⁷⁾. Previous study demonstrated that the calculated fiber number >5 μm in length required for inducing a 25% tumour risk differed between various fiber samples depending on fiber size and durability⁵⁾. The short glass fibers (median length 3.3 μm) required about tenfold number of fibers of the long glass fibers (median length 10.5 μm) for inducing a 25% tumour risk⁵⁾. This result suggests the similarity in the effect of fiber length between tumor risk in intraperitoneal studies and ability to induce O_2^- from macrophages *in vitro*.

In conclusion, the present method enabled comparison of induction of lucigenin-dependent CL from human monocyte-derived macrophages per fiber for mineral fibers of various sizes, and examination of the relationship between ability to induce CL and fiber size. Ability to induce CL increased with length, when samples were longer than approximately 6 μm .

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